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Cleavage without anchor addition accompanies the processing of a nascent protein to its glycosylphosphatidylinositol-anchored form

(endoplasmic reticulum/COOH-terminal signal peptide/transamidase)

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ABSTRACT **Rough microsomal membranes from most mammalian cells, in the presence of a translation system, process nascent proteins with appropriate COOH-terminal signal peptides to their mature glycosylphosphatidylinositol (GPI)-linked forms. The present study, using preprominiplacental alkaline phosphatase as substrate, shows that as much as 10% of the mature product is cleaved correctly but is not linked to GPI. Some of the factors that influence the relative proportions of GPI linked to free mini-placental alkaline phosphatase are the amounts of GPI in the cells and the amino acid substituent at the ω site of the nascent protein. A mechanism for explaining cleavage both with and without GPI addition is presented, which supports a transamidase type of enzyme as the catalyst.**

A nascent protein (preproprotein), prior to processing to a glycosylphosphatidylinositol (GPI) form, contains both NH_2- and COOH-terminal signal peptides that are cleaved sequentially to yield the mature form. Cleavage of the NH_2-terminal peptide follows translocation of the nascent protein into the endoplasmic reticulum and results in the proprotein. Cleavage of the COOH terminus from the latter, accompanied by attachment of the GPI moiety, yields the mature protein. We have investigated processing in cell-free systems using a shortened, engineered form of nascent placental alkaline phosphatase (PLAP) to which we have given the name preprominiPLAP (see Fig. 1). In studies with rough microsomal membranes (RMs) from normal and GPI-deficient cells, we presented evidence that GPI is an obligatory cosubstrate for COOH-terminal processing of nascent proteins (1). During those studies we noticed that, in addition to the major product of the reaction, mature GPI-linked miniPLAP, a minor product is also formed. From its migration on gels, we suggested that this could represent mature miniPLAP that was not GPI-linked. In this report, we present clear evidence that processing of prepromini PLAP in the endoplasmic reticulum to its mature GPI-linked form is accompanied by some cleavage at the ω site (the amino acid in a nascent protein that accepts the GPI moiety and, after cleavage, becomes the COOH-terminal residue of the mature protein) without GPI addition. The significance of this finding with respect to the mechanism of cleavage/GPI addition is discussed.

MATERIALS AND METHODS

Materials. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from *Bacillus thuringiensis* as described by Taguchi *et al.* (2). Radiolabeled compounds were from Amersham. All chemical reagents were from standard commercial sources unless otherwise indicated.

Cell Culture. All media were supplemented with 10% (vol/vol) fetal bovine serum. The CHO and HeLa cells were maintained at 37°C in a Biolafitte bioreactor in Iscove's modified Dulbecco's medium. COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine. L929 cells (from American Type Culture Collection) were maintained in M199 medium. The Ltk$^-$ and YH16/33 cells were grown in DMEM. The M31/25 cells from which M31/25-B4 and -C1 were derived were grown in RPMI 1640 supplemented with 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The L929 and YH16/33 are wild-type cells that produce GPI. Ltk$^-$ is a derivative of L929 that is deficient in synthesizing the GPI intermediate, *N*-acetylglucosaminephosphatidylinositol (3). The M31/25-B4 and -C1 cells are derivatives of YH16/33 that are deficient in transferring *N*-acetylglucosamine to phosphatidylinositol (4). YH16/33, M31/25, and Ltk$^-$ were gifts from E. T. H. Yeh (Texas Biotechnology, Houston).

Preparation of RMs. For the preparation of RMs all steps were carried out on ice or at 4°C. Cell pellets were washed once with PBS and once with 10 mM triethanolamine (TEA; pH 8.0) prior to use. For RM preparation from 50- or 100-liter cultures, 200 g of cells was brought to 300 ml with ice-cold 10.0 mM TEA (pH 8.0), and an additional 300 ml of ice-cold 0.5 M sucrose/10.0 mM TEA, pH 8.0 was added to give a final volume of 600 ml. For smaller preparations, cell pellets (5 g) were resuspended in a total volume of 30 ml. The cell suspension was placed in a cell-disruption bomb (Parr Instruments, Moline, IL) that was pressurized with nitrogen to 1200–1500 psi (1 psi = 6.89 kPa) and placed on a stirrer for 1.5 hr. After discharge from the bomb, the lysate was treated as described by Aronson and Touster (5), and the microsomal pellet was resuspended in 250 mM sucrose/50 mM TEA, pH 7.5 to a final concentration of 50 A_{280} units/ml. Stock solutions of RMs were frozen on dry ice and stored at −70°C.

Preparation of mRNA. After linearization of each miniPLAP plasmid with *Hin*dIII, the DNA was transcribed using a Riboprobe system II kit (Promega), and the mRNA was purified using an RNaid kit (Bio 101). The Δ179 truncation construct, prepared as described (6), contained the codon for aspartate at its COOH terminus (which corresponds to the ω site in prepromini PLAP).

Cotranslational Processing of miniPLAP. Unless otherwise stated, translations were carried out with mRNA representing prepromini PLAP 208 $^{\omega}$Ser using rabbit reticulocyte lysate (Promega) in 25-μl reaction volumes, as described (6). RMs from each cell type were added to a final concentration of ≈8 A_{280} units/ml.

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Abbreviations: GPI, glycosylphosphatidylinositol; PLAP, placental alkaline phosphatase; RM, rough microsomal membrane; PI-PLC, phosphatidylinositol-specific phospholipase C; ω site, the amino acid in a nascent protein that accepts the GPI moiety and, after cleavage, becomes the COOH-terminal residue of the mature protein.

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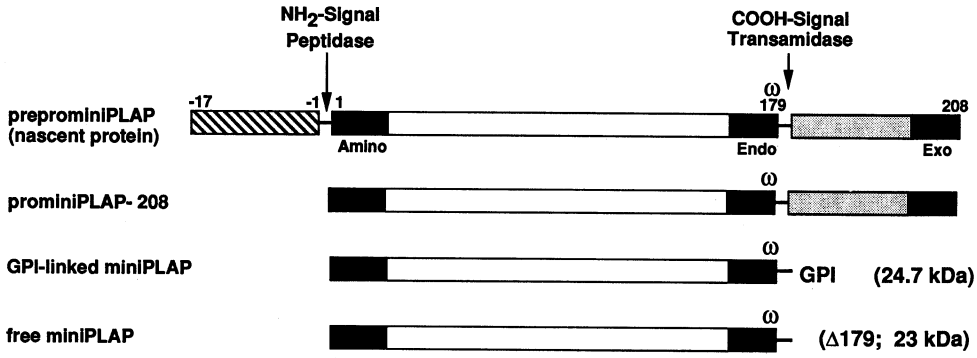

FIG. 1. Steps in the processing of prepromini PLAP in RMs. The black boxes represent epitope sites for antisera that were used to monitor the intermediate and final products of processing. The amino antibody does not react with its epitope when it occurs as an internal sequence, as in prepromini PLAP. Cleavage by the NH_2-terminal signal peptidase to promini PLAP places the epitope at the terminus where it is now reactive with its antibody. The epitope to the endo antibody ends in residue 179, the ω site, which remains as the COOH terminus in the mature forms of mini PLAP. The exo antibody is part of the COOH-terminal signal peptide site and is lost during processing to the mature forms.

Phase Separations. For these studies, translation of mini-PLAP-208 $^{\omega}$Ser and mini PLAP Δ179 mRNA were carried out as described above but were scaled up to 100 μl. Triton X-114 was precondensed as described by Bordier (7). Treatment with PI-PLC and phase separations were carried out as described (6).

Immunoprecipitation and SDS/PAGE. After incubation, samples were diluted in 10% SDS/8% 2-mercaptoethanol (1:1, vol/vol) and boiled for 5 min. After cooling to room temperature, an equal volume of water was added to each sample and an aliquot was removed for immunoprecipitation, as described (8). Unless otherwise stated, affinity-purified rabbit polyclonal IgG from Accurate Chemicals was used. SDS/PAGE was performed as described (9, 10) with the following modifications. The 15% resolving gels were extended from 12.0 to 15.0 cm and were run at constant power (15 W per gel for 3.5 hr) instead of constant current. Visualization and quantitation were performed using a PhosphorImager (Molecular Dynamics).

RESULTS

As shown in Fig. 1, the processing of nascent prepromini-PLAP-208 (28 kDa) gives rise to promini PLAP (27 kDa) in which only the NH_2-terminal signal sequence has been removed. The latter is converted to GPI-linked (mature) mini PLAP (≈24.7 kDa) in which a 29-residue COOH-terminal peptide signal sequence has been replaced by a GPI-anchor attached to residue 179. In the present study we show that in RMs prepared from all cell lines that we investigated, including those that are deficient in GPI synthesis, there was an additional protein formed that migrated as a band with an apparent mass of ≈23 kDa (Fig. 2).

Based on its apparent molecular mass on SDS/PAGE gels, the 23-kDa band could have been the product of cleavage of the NH_2- and COOH-terminal signal peptides but without the addition of the GPI anchor. We, therefore, compared the products of processing of prepromini PLAP-208 to those of the Δ179 mutant. The latter is a truncation mutant that, after NH_2-terminal processing, yields a product that terminates at the same ω site, representing mature mini PLAP but without the GPI moiety (1). In cotranslational experiments performed with RMs from HeLa and COS cells, the 23-kDa band obtained by processing of prepromini PLAP-208 comigrated with the NH_2-terminally processed band from the Δ179 construct (Fig. 3). Furthermore, in all experiments the 23-kDa protein appeared as a sharp band, indicating that it represents a single product rather than cleavage by nonspecific proteases.

To further identify the products of COOH-terminal processing of prepromini PLAP, we used several site-specific antibodies (see Fig. 1). As shown in Fig. 4, the immunologic profile of the 23-kDa protein was the same as for mature GPI-linked mini PLAP. Like GPI-linked mini PLAP, it had lost the exo epitope, indicating cleavage of the COOH-terminal signal peptide. Reaction with the NH_2 antibody indicates that both have the same NH_2 terminus; reaction with the endo antibody indicates termination at the same ω site. The presence of epitopes in the 23-kDa protein that react with the NH_2 and endo antibodies represents further evidence that it is not a random proteolytic product but a specific product of processing related to mature GPI-linked mini PLAP.

The difference in molecular mass between the two, ≈1.7 kDa, agrees well with the calculated mass of GPI (11, 12). To determine whether the 23-kDa product contained a GPI residue, we carried out a phase-distribution study. As shown in

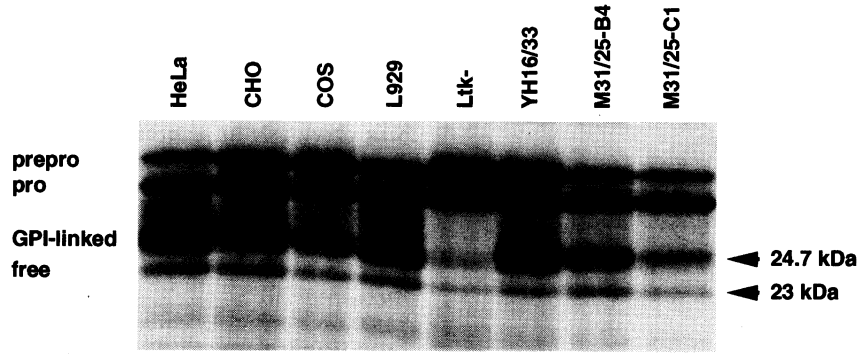

FIG. 2. Processing of prepromini PLAP-208 $^{\omega}$Ser by RMs from various cell types. HeLa, CHO, COS, L929, and YH16/33 contain functional GPI; Ltk$^-$, which was derived from L929, and M31/25-B4 and -C1, which were derived from YH16/33, are defective in GPI production. All samples were immunoprecipitated and subjected to SDS/PAGE.

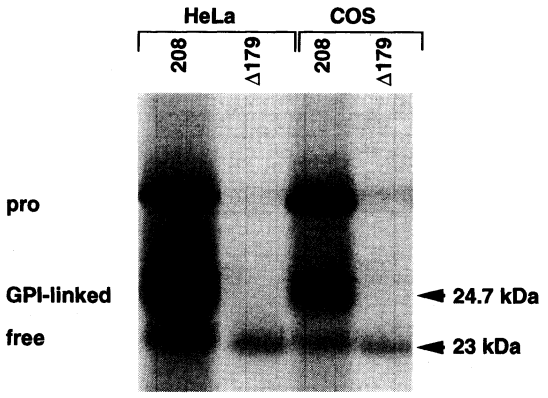

FIG. 3. Cotranslational processing of mRNAs for prepromini-PLAP-208 $^{\omega}$Ser (208) and preprominiPLAP Δ179 $^{\omega}$Asp (Δ179). After incubation, samples were immunoprecipitated with the NH_2 antibody, which does not react with the prepro forms.

Fig. 5, mature miniPLAP (24.7 kDa) distributed itself equally between the aqueous and the detergent phases. This is characteristic of GPI-linked proteins. By contrast, the 23-kDa protein appeared mainly in the aqueous phase, indicating that it is devoid of a lipid component. After treatment with PI-PLC, which removes the lipid portion of GPI, far less of the 24.7-kDa protein appeared in the detergent phase (data not shown). PI-PLC had no effect on the 23-kDa protein.

As shown in Fig. 2, RMs from all the cells that we investigated processed prominiPLAP to both the GPI-linked and the free form. However, the total amount processed (GPI-linked plus free) varied considerably. RMs from HeLa and CHO cells were the most active, and RMs from GPI-deficient mutant T cells were the least active. It is of interest, however, that as the total amount of COOH-terminal processing decreased the relative proportion of the free product increased. As shown in Fig. 6, in RMs from most wild-type cells the free product accounted for ≈10% of the total. By contrast, in RMs from GPI-deficient cells, as much as 50% of the COOH-terminally processed material was not linked to GPI.

Another unexpected finding was that the relative amount of the free protein produced depended on the amino acid substituted at the ω site of the nascent protein. It has been shown that only the six amino acids glycine, alanine, serine, cysteine, aspartate, and asparagine serve effectively at the ω site for cleavage and GPI addition (13–16). The above studies were all carried out with prominiPLAP-208 $^{\omega}$Ser from which the largest amounts of the free protein are produced. As shown in Fig. 7, under conditions where all five of the ω site mutants that were studied yielded the same amount of GPI-linked miniPLAP, the $^{\omega}$Ser mutant yielded, by far, the largest amount

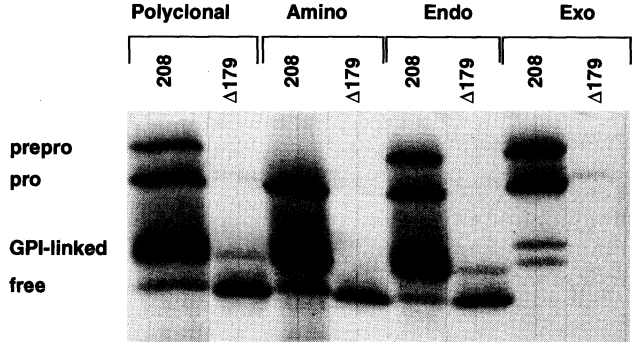

FIG. 4. Immunologic profile of the products formed on processing of mRNAs for prepromini PLAP-208 $^{\omega}$Ser (208) and preproPLAP Δ179 $^{\omega}$Asp (Δ179). After incubation, each sample was divided into four aliquots for immunoprecipitation with one of the four antibodies.

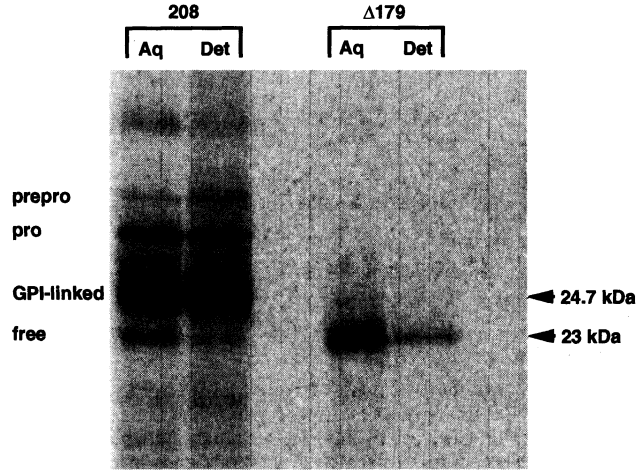

FIG. 5. Phase distribution. After incubation, samples were allowed to distribute between Triton X-114 (Det) and water (Aq). Aliquots from each phase were then immunoprecipited with polyclonal antibody and subjected to SDS/PAGE.

of the 23-kDa free protein forms. Smaller amounts were obtained with the $^{\omega}$Gly and $^{\omega}$Asp mutants and even less with the $^{\omega}$Ala and $^{\omega}$Cys forms.

DISCUSSION

The present studies clearly show that processing of a protein to its mature GPI-linked form is accompanied by some conversion to the mature free form. At first glance, this might bring into question whether a transamidase is indeed the catalyst for simultaneous cleavage and GPI addition or whether cleavage and GPI addition are catalyzed by three distinct enzymes—i.e., a signal peptidase, an enzyme to activate the newly exposed ω COOH group, followed by a condensing enzyme. As shown below, our findings are, however, entirely compatible with a transamidase type of mechanism and actually represent supporting evidence for it.

If one wishes to interpret our findings as indicating cleavage by a signal peptidase as an independent first step, then one must explain why RMs from GPI-deficient cells do not produce free mature miniPLAP in lieu of GPI-linked miniPLAP. As shown in Figs. 1 and 6, GPI-deficient RMs produce far less of both GPI-linked and free miniPLAP. In fact, in an earlier report with RMs that were completely devoid of GPI†, no GPI-linked or free miniPLAP were produced (1). Interestingly, in those studies addition of GPI-enriched extracts to the completely depleted RMs restored production of both the GPI-linked and free forms in the same proportions as were produced by the GPI-competent progenitor cells (i.e., about 10% free miniPLAP). Thus, GPI is required for the production of both GPI-linked miniPLAP as well as for free miniPLAP.

Transamidases and transpeptidases catalyze the cleavage of an amide bond in a protein and simultaneously activate what was previously an internal carbonyl group. In the process, a peptide is eliminated. Concomitant with this, another peptide or primary amine is attached to the newly formed carbonyl group. The mechanism proposed for this (17, 18) involves an intermediate enzyme–substrate complex in which a highly

†RMs from the original sample of M31/25 cells that we received in 1991 were essentially devoid of all GPI processing activity (1). The cells were kept frozen until they were grown up again for the present studies. RMs from the recently cultured M31/25 cells contain significant amounts of functional GPI, but the cells are not clonally homogeneous as shown by the different degrees of processing of the two subclones, M31/25-B4 and M31/25-C1.

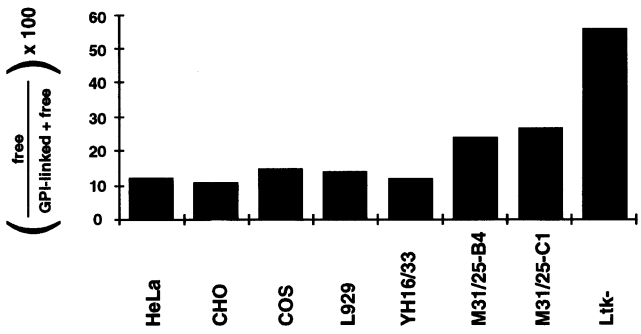

FIG. 6. Relative proportions of GPI-linked and free miniPLAP produced by various cells. The PhosphorImager was used to obtain quantitative data from the gels shown in Fig. 2. The three cell lines on the right are deficient in GPI production.

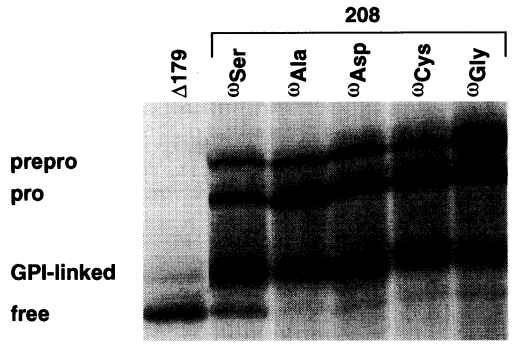

FIG. 7. Influence of the ω substituent in preprominiPLAP-208 on the production of free miniPLAP. After incubation, aliquots of each sample were taken so as to contain approximately the same amount of GPI-linked miniPLAP. Immunoprecipitation and SDS/PAGE were carried out as before.

reactive carbonyl is formed. In the case of prominiPLAP, it is such an enzyme-activated intermediate that accepts the nucleophilic amino group of the ethanolamine residue of GPI. Such an intermediate would be expected to form even in the absence of GPI and react with an abundant nucleophile such as water to yield free mature miniPLAP. The fact that GPI is apparently an absolute requirement for processing to both GPI-linked as well as free miniPLAP argues for a role for GPI in addition to its role as a cosubstrate. In a previous report (1), we suggested that it is a membrane-bound transamidase–GPI complex to which prominiPLAP binds rather than to the free transamidase. This would explain why GPI is necessary even for the formation of free miniPLAP.

The unusually large amounts of free miniPLAP formed from the ωSer mutant, again only in the presence of GPI, lend further support to a transamidase–GPI–prominiPLAP complex. Of the six amino acids that can serve effectively at the ω site, only serine can form a six-membered ring as part of the complex. Initial enzymatic attack at the amide function between Ser^{179} and Ala^{180} (Fig. 8 *Top*) can result in the formation of a partially stabilized six-membered intermediate (Fig. 8 *Middle*). Such an intermediate would undergo rapid interaction with the NH_2 group of GPI to give the GPI-linked protein as the major product. However, the greater stability of the serine intermediate may permit appreciable time for hydrolysis to occur via pathway **b** (Fig. 8 *Bottom*) as a competing side reaction. When Ser^{179} is replaced by one of the other amino acids (which would not result in stabilized intermediates), this hydrolytic side reaction would be minimized and pathway **a** would predominate.

While the present studies clearly support a transamidase as catalyzing COOH-terminal processing of precursors to GPI proteins, they point to problems that will be faced in attempts to purify the putative transamidase. Reaction of the intermediary enzyme–substrate complex with water to yield free mature miniPLAP suggests that stronger nucleophiles, such as hydrazine and hydroxylamine, could bypass the need for GPI. Both of these reagents are known to react with activated carbonyl groups in transamidase type reactions (18). However, if GPI acts not only as a cosubstrate but also allosterically, then reconstitution of the enzyme even in the presence of strong nucleophilic reagents will still require GPI. Unfortunately, the latter has yet to be obtained in pure form in amounts required

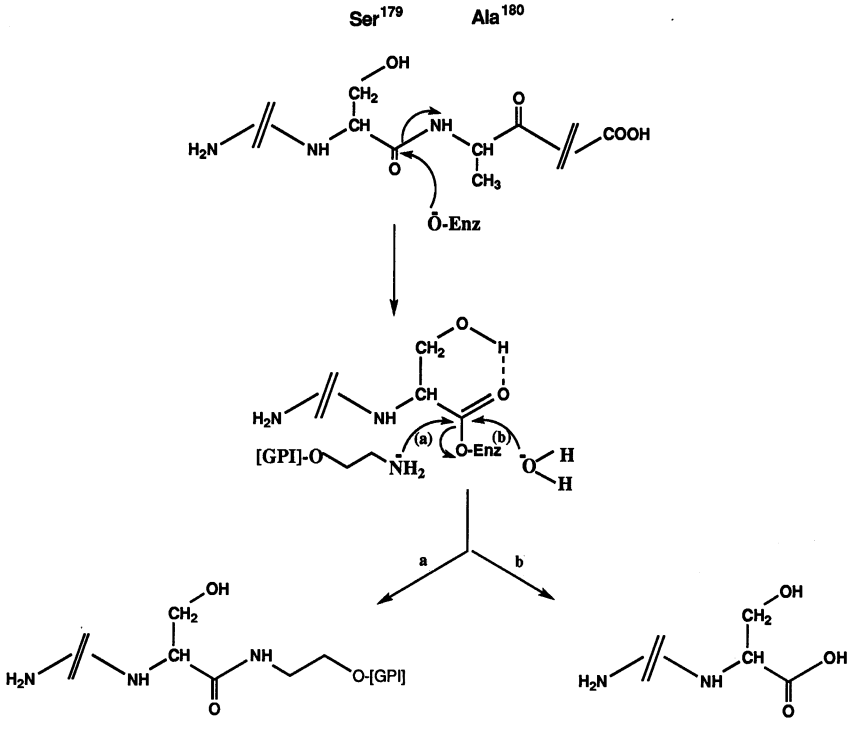

FIG. 8. Proposed mechanism of cleavage of prominiPLAP-208 ωSer by the putative transamidase to yield both GPI-linked and free miniPLAP.

for enzyme studies. However, it may be possible to isolate the transamidase as the GPI complex.

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